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METHEMOGLOBINEMIA DUE TO DEFICIENCY OF DPNH DIAPHORASE

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METHEMOGLOBINEMIA DUE TO DEFICIENCY OF DPNH DIAPHORASE

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Methemoglobinemia is usually discovered because of the peculiar cyanotic appearance of an individual, which is not explainable in terms of accompanying circulatory or respiratory distress. The skin color resembles that found in anoxic cyanosis, but tends to be somewhat more grayish.

The diagnosis is confirmed by testing for the presence of methemoglobin in the patient's blood by a colorimetric method such as that of Evelyn and Malloy¹. A simpler way which requires no instruments is as follows: Add 1 part of the patient's blood to 20 parts 0.9 % NaCl, shake, and compare with a similar suspension of normal red cells. If the patient is methemoglobinemic, the cells in suspension will appear to be browner than the control. If a drop of neutral cyanide is then added to the suspensions, the tubes will appear nearly identical in color*.

Having established that methemoglobin is present, the next problem is to determine the cause for its presence.

Methemoglobin is the oxidized form of hemoglobin; that is, the iron in the heme is in the ferric (Fe^{+3}) form in methemoglobin instead of the ferrous (Fe^{+2}) form found in hemoglobin. In the presence of oxygen pure hemoglobin is unstable, and will slowly oxidize to methemoglobin. Certain drugs and other compounds, including nitrite, change the structure of hemoglobin and speed up its oxidation. When these chemicals are introduced into the body, *toxic methemoglobinemia* is produced. The only sure way of diagnosing toxic methemoglobinemia is to identify

(*) In methemoglobinemia due to some types of hemoglobin M, the brown color will not disappear in the presence of cyanide.

and remove the offending agent, whereupon the methemoglobin disappears.

Certain abnormal hemoglobins are structurally altered so that ferric iron in the molecule can not normally be reduced to ferrous iron. These hemoglobins are known collectively as hemoglobins M, and result in a typical methemoglobinemia. The presence of hemoglobin M can be detected by electrophoresis of an oxidized hemolyzate at pH 7.0 in starch gel, since all known hemoglobin M specimens show an abnormal component under these conditions.

It is evident from the above that a mechanism is present in normal cells which can reduce methemoglobin, and which keeps hemoglobin in the ferrous form. The third type of methemoglobinemia results when this mechanism is non-functional. That such a deficiency exists was first shown in 1945². If one treats red cells with nitrite, washes them thoroughly and incubates with glucose, there is a marked difference between normal and methemoglobinemic cells in the rate at which methemoglobin is reduced (Table I).

TABLE I. — *Rate of reduction of methemoglobin in whole cells and under conditions simulating those in the human red cell.*^{4 12}

Reducing system	First Order Rate min ⁻¹	Percent Reduced Per Hour	Percent of Reducing Capacity
DPNH Diaphorase	5.8×10^{-4}	8.0	61
Ascorbic Acid	1.5×10^{-4}	2.1	16
Glutathione	1.2×10^{-4}	1.6	12
TPNH Diaphorases	0.5×10^{-4}	0.7	5
DPNH Dehydrogenase II	0.6×10^{-4}	0.8	6
Total	9.6×10^{-4}	13.2	100
Whole Cells		10-15	
Methemoglobinemic Cells		2-5	

A much simpler way of diagnosing methemoglobinemia due to enzymatic deficiency is based on the ability of normal hemolyzates to catalyze the reduction of 2,6-dichlorophenolindophenol by reduced diphosphopyridine nucleotide (DPNH) (Figure 1). With a single exception this catalytic capacity is completely absent in all reported cases of methemoglobinemia due to enzyme deficiency³.

The amount of methemoglobin found in the cells of individuals with enzyme deficiency ranges between 7 and 45 percent of the total hemoglobin. Starch electrophoresis shows that the cells contain a mixture of fully reduced $[(\text{Fe}^{+2})_4]$ and half-oxidized $[(\text{Fe}^{+2})_2(\text{Fe}^{+3})_2]$ hemoglobin. This type of methemoglobinemia has been found throughout the world — in Caucasians, Asiatic groups and North American Indians — but it as yet unknown in Negroes. A particularly high prevalence has been found in three North American groups, in Southern Eskimos and Atha-

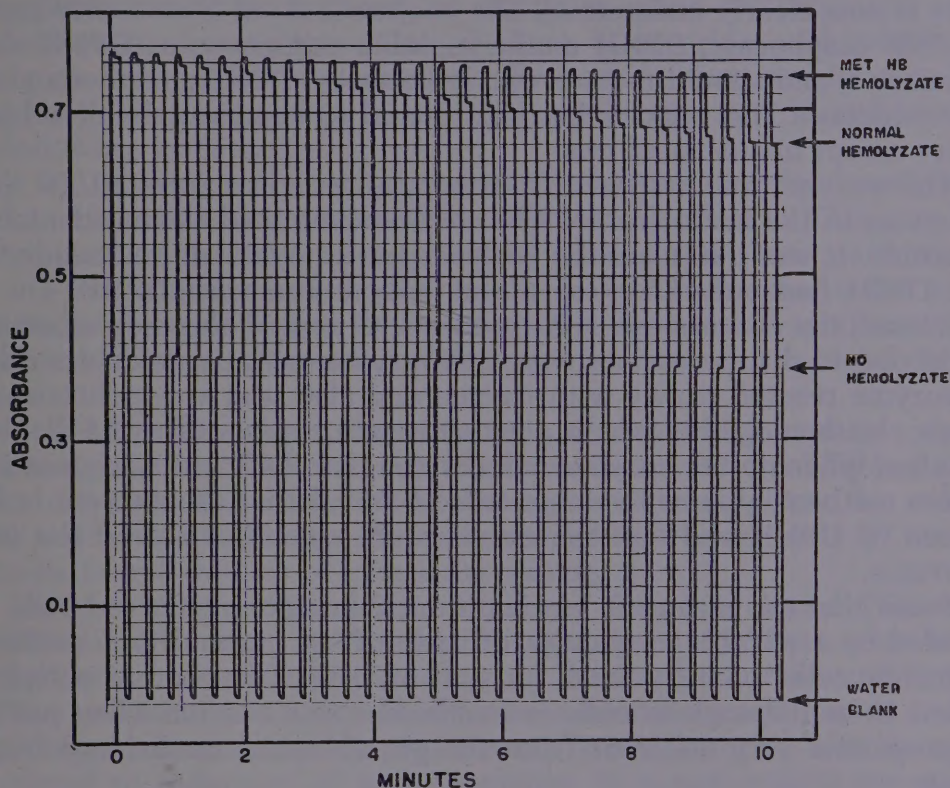


FIG. 1. — Spectrophotometric tracing at 600 $m\mu$ of reduction of 2,6-dichlorophenolindophenol by normal and methemoglobinemic hemolyzates.

baskan Indians in Alaska, and in Navajo Indians in the southwestern United States ^{4 5}.

Ordinarily, there is no clinical evidence of abnormality in these persons other than cyanosis but there may be a compensatory polycythemia. Since methemoglobin can not bind oxygen it has no respiratory effectiveness. In methemoglobinemia, the erythropoietic tissues are stimulated to

produce a sufficient amount of active hemoglobin, regardless of the amount of methemoglobin present.

A significant proportion of methemoglobinemic persons in certain families are also deficient mentally. Recently, Fialkow et al⁶ have pointed out that these all have occurred in families with methemoglobinemia due to enzyme deficiency. They suggest that methemoglobinemia may cause a tendency toward cerebral hypoxia which makes the individual more susceptible to factors causing mental deficiency, although not necessarily producing such deficiency in every case.

The biochemical basis of methemoglobinemia due to enzyme deficiency is now clearly understood. The enzyme lacking is variously known as DPNH diaphorase, DPNH methemoglobin reductase, or DPNH dehydrogenase I, and should most properly be called DPNH: methemoglobin oxidoreductase. This enzyme has been found to be absent in all subjects studies except one isolated case.

This enzyme was prepared from normal cells in a form 50,000 times as pure as in the hemolyzate⁷. The enzyme contained flavin adenine dinucleotide. It was quite specific with respect to pyridine nucleotide, and with TPNH had only 1.5 % of the activity found with DPNH. On the other hand, the enzyme was non-specific with regard to other substrates. In addition to diaphorase activity with various oxidation-reduction dyes, the enzyme reacted slowly with oxygen and also was a cytochrome-c-reductase. Methemoglobin was a poor substrate compared to 2,6-dichlorophenolindophenol; the enzyme reduces the dye 9000 times as fast as it reduces methemoglobin. Evidence was obtained that the enzyme is first reduced by DPNH and that the enzyme subsequently reduced the other substrates.

Proof that the enzyme is lacking in methemoglobinemic red cells was provided by a parallel enzyme purification from normal and methemoglobinemic cells⁸. A very small amount of DPNH diaphorase activity is present in methemoglobinemic red cells, but this enzyme when purified has properties very different from the physiological methemoglobin reductase.

The classical genetics of enzyme deficiency methemoglobinemia are now also well understood. Methemoglobinemia in an autosomal recessive character, and obligatory heterozygotes seldom if ever show any appreciable methemoglobin in red cells. The heterozygotes do, however, show about half the normal level of DPNH diaphorase activity⁹. Thus it is easy to trace inheritance within a family tree.

The locus of the trait on the human chromosomes is unknown. It is not linked closely with any of the blood group systems ABO, MNS, RH, Duffy, Kidd or P¹⁰, nor with the serum factors haptoglobin and Gc, nor with the red cell enzymes acid phosphatase and phosphoglucomutase.

The nature of the mutational change in the enzyme is also not known. Conceivably, this could be an amino acid substitution so that the enzyme could no longer bind flavin, or would otherwise be made catalytically inactive, or it may be a change in a control gene. The one case known where methemoglobin reductase activity was lacking but DPNH diaphorase activity was normal¹¹ may be the result of a mutation causing a conformational change so that the larger methemoglobin molecule is not a substrate, while the smaller dye molecule is.

It is clear however that DPNH diaphorase can not be the only system that reduces methemoglobin in the red cell. Otherwise, all of the hemoglobin would be oxidized. At least five other systems exist which can reduce methemoglobin. One of these is ascorbic acid, which will reduce methemoglobin nonenzymatically both *in vitro* and *in vivo*. That ascorbic acid is important in reducing methemoglobin is indicated by the following evidence:

a) In many early studies, ascorbic acid levels in blood were measured and found to be low in methemoglobinemic subjects. This may have contributed to the early recognition of cyanosis in these patients.

b) In Alaska, ascorbic acid intake may be near zero in the winter months, and much higher in summer. The amount of methemoglobin in our subjects also fluctuated with season and was much higher in winter⁴.

c) Ascorbic acid is the treatment of choice for cyanosis due to methemoglobinemia. After two or three weeks of therapy (250 mg ascorbic acid per day) the level of methemoglobin in blood can be reduced to 10 to 15 percent. Unless the methemoglobin level exceeds 20 percent, cyanosis is not evident to casual inspection.

Ascorbic acid at the level commonly found in blood (about 1.0 mg/100 ml) will reduce methemoglobin at a slow rate. There is no known mechanism in red cells for reducing the oxidized ascorbic acid formed, but the cell membranes are freely permeable to ascorbic acid, and the supply could be continuously replenished. While ascorbic acid seems to be related to reduction of methemoglobin, it is not critical for it. Certainly, methemoglobinemia is not found in scurvy.

All other known mechanisms of methemoglobin reduction are dependent ultimately on the metabolism of glucose. Glucose can be metabolized in two ways by the glycolytic route or by the hexose monophosphate shunt. Glycolysis results in the reduction of DPN to DPNH and the shunt results in conversion of TPN to TPNH. Since red cells have no appreciable oxygen uptake, the bulk of the pyridine nucleotides are normally in the reduced form.

The second mechanism for reducing methemoglobin involves glutathione. This substance is present in considerable amount in red cells

and at this concentration will reduce methemoglobin nonenzymatically at a slow rate. Glutathione will also react slowly with oxygen, and it is kept in the reduced form by the enzyme glutathione reductase. This reduction is accomplished by TPNH although DPNH may be active to some extent. No disorder in glutathione metabolism is known that will produce methemoglobinemia.

A third system that will reduce methemoglobin is a second DPNH dehydrogenase that is present in minor amounts in both methemoglobinemic⁸ and in normal¹² red cells. This enzyme is as effective as DPNH diaphorase in reducing methemoglobin but is present in red cells in only about one-tenth the amount. Consequently the second DPNH dehydrogenase is of only minor importance in keeping hemoglobin reduced.

There are at least two TPNH dehydrogenases present in human red cells. These have been purified and their properties studied¹². The two enzymes are closely similar in catalytic properties, but are separable by chromatography. The TPNH dehydrogenases are poor methemoglobin reductases, being 55,000 times as active in reducing 2,6-dichlorophenolindophenol as in reducing methemoglobin. They are not flavin enzymes. The TPNH dehydrogenases do not appear to be of major importance in methemoglobin reduction. In all cases of methemoglobinemia studied thus far, the level of TPNH diaphorase has been found normal.

The relative effectiveness of these enzymes at their normal concentration in human red cells has been evaluated (Table I). A comparison of these rates shows that the DPNH diaphorase, absent in methemoglobinemia, should account for 61 percent of the reducing capacity of the normal red cell. This result is in good agreement with rates of reduction found in normal and methemoglobinemic red cells, where the latter have lost three-fourths of their reducing capacity.

We know that when DPNH diaphorase is absent, two-thirds of the methemoglobin reducing capacity of the red cell is lost, and methemoglobinemia results. In obligatory heterozygotes with partial DPNH diaphorase deficiency, one-third of the reducing capacity is lost, but no additional methemoglobin is found. Since the other systems in the red cell which reduce methemoglobin together constitute less than a third of the reducing capacity, we may conclude that absence of any one of these would not produce methemoglobinemia. In fact, no such cases are known. The only enzymatic deficiencies that could be expected to produce methemoglobinemia are absence of DPNH diaphorase, deficiency of DPN, or the absence of an enzyme necessary for reduction of DPN to DPNH.

Prognosis of the disease is good, and it appears to have no harmful physiologic effects, nor does it affect longevity. Therapy is given usually only for cosmetic reasons, and consists of methylene blue or ascorbic

acid. Methylene blue is rapidly reduced by glutathione or TPNH diaphorase to leukomethylene blue. Leukomethylene blue will in turn rapidly reduce methemoglobin to hemoglobin. This reaction is reversible; and in the absence of reducing agents, methylene blue will quickly oxidize hemoglobin to methemoglobin. As mentioned above, ascorbic acid is usually preferred for treatment.

REFERENCES

1. EVELYN, K. A. and MALLOY, H. T.: Microdetermination of oxyhemoglobin, methemoglobin and sulfhemoglobin in a single sample of blood. *J. Biol. Chem.*, 126, 655, 1938.
2. BARCROFT, H., GIBSON, Q. H., HARRISON, D. C. and McMURRAY, J.: Familial idiopathic methaemoglobinemia and its treatment with ascorbic acid. *Clin. Sci.*, 5, 145, 1945.
3. SCOTT, E. M., and GRIFFITH, I. V.: The enzymic defect of hereditary methemoglobinemia: diaphorase. *Biochem. Biophys. Acta*, 34, 585, 1959.
4. SCOTT, E. M., and HOSKINS, D. D.: Hereditary methemoglobinemia in Alaskan Eskimos and Indians. *Blood*, 13, 195, 1958.
5. BALSAMO, P., HARDY, W. R. and SCOTT, E. M.: Hereditary methemoglobinemia due to diaphorase deficiency in Navajo Indians. *J. Pediatrics*, 65, 928, 1964.
6. FIALKOW, P. J., BROWDER, J. A., SPARKES, R. S. and MOTULSKY, A. G.: Mental retardation in methemoglobinemia due to diaphorase deficiency. *New England J. Med.*, 273, 840, 1965.
7. SCOTT, E. M. and MCGRAW, J. C.: Purification and properties of diphosphopyridine nucleotide diaphorase of human erythrocytes. *J. Biol. Chem.*, 237, 249, 1962.
8. SCOTT, E. M.: Purification of diphosphopyridine nucleotide diaphorase from methemoglobinemic erythrocytes. *Biochem. Biophys. Res. Commun.*, 9, 59, 1962.
9. SCOTT, E. M.: The relation of diaphorase of human erythrocytes to inheritance of methemoglobinemia. *J. Clin. Invest.*, 39, 1176, 1960.
10. SCOTT, E. M., LEWIS, M., KAITA, H., CHOWN, B. and GIBLETT, E. R.: Absence of close linkage of methemoglobinemia and blood group loci. *Amer. J. Human Genet.*, 15, 493, 1963.
11. TOWNES, P. L., and LOVELL, G. R.: Hereditary methemoglobinemia: a new variant exhibiting dominant inheritance of hemoglobin A. *Blood*, 18, 18, 1961.
12. SCOTT, E. M., DUNCAN, I. W. and EKSTRAND, V.: The reduced pyridine nucleotide dehydrogenases of human erythrocytes. *J. Biol. Chem.*, 240, 481, 1965.

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